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THE TUMOR-ASSOCIATED INFLAMMATION DETERMINES EMT-STEMNESS IN GLIOMA MALIGNANCY

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ABSTRACT

Recent data have expanded the conception that cancer-related inflammation is a crucial component of tumor progression. Indeed, anti-inflammatory therapies have shown efficacy in cancer prevention and treatment. It`s becoming clear that blocking inflammation will play a major role in cancer outcome. Every tumor stage may be potentiated by the underlying inflammatory process. But an exact mechanism of tumor-associated inflammation in cancer metastatic progression is not clear. The investigation of the mechanisms of the relationship between cancer progression and inflammation remain elusive. In this study, we have focused on the causal interaction between tumor-associated inflammation and EMT-stemness stage during glioma malignancy. We found that verapamil Ca-blocking drug with accumulating anti-inflammatory function in glioma therapies targeted EMT-inducer transcription factor Snail gene expression in malignancy glioma patients along with anti-inflammatory actions as increasing blood cells transmembrane potential (TMP) and decreasing lymphocytes blast transformation proliferation (LBTP) in the primary blood cell culture assay. On the date obtained we have proposed that glioma-related inflammation determines EMT-stemness phenotype development in glioma malignancy. In conclusion, our results have revealed that anti-inflammatory therapy as verapamil may be efficacy in the targeted EMT-stemness in cancer metastatic progression and combining current anti-inflammatory drugs with the targeting EMT-stemness pathway may emerging improve metastatic patient treatment and cure.

KEYWORDS: Glioma Malignancy, Cancer-Related Inflammation, EMT-Stemness, Verapamil.

INTRODUCTION

The inflammation is a critical component of tumor progression: inflammatory responses play decisive roles at different stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis.[1-5] The causal link between inflammation and cancer has been established more than a century ago by Rudolf Virchow, who noticed the infiltration of leukocytes in malignant tissues, has recently found a number of genetic and molecular confirmations.[6] So, tumors that are not epidemiologically linked to pathogens are characterized by the presence of an inflammatory component in their microenvironment.[7] Therefore, cancer-associated inflammation is recently recognized the seventh hallmark of cancer.[8] Indeed, anti-inflammatory therapies have efficacy provided for the use of cytokine and chemokine blockade in the chemoprevention and treatment of malignant diseases.[9]

The primary tumor-related inflammation is resulted fromboth cancer cells and stromal components in tumor microenvironment wich actively interacte with each other to participate in tumor progression.[10] The tumor cells have co-opted some of the signalling molecules such as chemokines, cytokines and growth factors, as well as their receptorsand reactive oxygen species (ROS) that have been implicated in the etiology of inflammation-associated cancers.[10,12] Therefore, inflammation underlies the plasticity of tumor microenvironment using in particular the surrounding host stromal cells (as fibroblasts, immune cells, endothelial cells, extra cell matrix and the bone marrow-derived cells in providing cells for the stroma) eventually promoting tumor proliferation, survival and migration,invasion and metastasis of malignant cells.[13] Recently is becoming clear that the tumour microenvironment, which is largely orchestrated by inflammatory cells and inflammatory mediatorsis an indispensable participant in the neoplastic process.[14] These insights are fostering new anti-inflammatory therapeutic approaches to target influence tumor microenvironmet in cancer progression.[15] Together, cancer-associated inflammation has effects on the ability of cancers to metastasize, on the clinical manifestations of cancer, and on the ability of the patient to tolerate anticancer therapy, but the molecular link mechanisms

between inflammation and cancer metastasizing continue to be elucidated. Recent evolving understanding of tumor-associated inflammation binding with cancer stemm cell biology responciblefor the initiation, growth, metastasis, therapy resistance and recurrence of cancers and the potential for effectively treating patients.[16] It is advantaged that the factors associated with chronic inflammation, including cytokines, oxidative stress, and hypoxia, induce the activation of specific cellular response programs that can affect the survival, proliferation, metabolism, and differentiation of cancer cells with invasive-metastatic potentials.[17] But the notion that inflammation plays in cancer stemm cell progression are slowly being elucidated and a better understanding of the molecular mechanisms between tumor-associated inflammatory and CSCs will provide invaluable diagnostic, therapeutic and prognostic targets for clinical application. However, the mechanisms linking inflammation and stemness expression in cancer progression as well as glioma malignancyremainelusive. In cancer biology, EMT (epithelial-to-mesenchymal transition) is one mechanism to explain the invasive and migratory capabilities that epithelial carcinomas acquire during metastasis.[18,19] Ourstudy is focused onthe origin inflammation-induced EMT-stemness mechanismin cancer stem cellglioma malignancy.

MATHERIALS AND METHODS

Lymphocyte proliferative activity determination.

The primary peripheral blood cell culture was investigated from28 patients withmalignant gliomas (IV stage). Anti-inflammatory calcium blocker verapamil drug was used in lymphocyte culture treatment. Modification of lymphocytes blasttransformation reaction (LBTR) was realized *in vitro* by application of 0,25% verapamil solutions (Pharmak). Solutions make ready in subsidiary dilutions from 10-1 to 10-5 times immediately before 72 hours blood cells cultivation in RPMI medium. 2 ml of RPMI medium, 600 mcl of blood cells without plasma, 60 mcl of different concentrations of verapamil, 60 mcl of phytohemagglutinin (PHA) (Sigma, 1mg/ml H**2**0) and 20 mcl of antibiotic was put into each 2-cm Petri dishes.

Transmembrane potential value by theblood cells aggregation determination.

Transmembrane potential (TMP) level model on blood cells membrane mediates by blood cells aggregation level indices. New method for blood cells aggregation level was determined at malignant gliomas by use of ultrasensitive instruments based on surface plasmon resonance phenomenon (SPR).[20] The application of the new method becomes the possibility to determine objective data without use of buffer systems or salt solutions that can influence on blood cells aggregation levels. The highest possible SPR signal was taken on blood cells without plasma. SPR unit is the laser angle of deviation, that measured in relative numbers and converting in percentages.

Determination ofmRNASnail gene expression by real-time reverse transcription–polymerase chain reaction.

RNA extraction from cultured blood cells was performed using TRIzol reagent (Invitrogen) per manufacturer's instructions. Three hundred nanograms of RNA were reverse transcribed to cDNA using iScript Reverse Transcription Supermix for quantitative real-time polymerase chain reaction (qRT-PCR) (BioRad Laboratories). PCR was performed using the Biorad CFX96 Real-Time PCR Detection System (BioRad Laboratories) machine with the SsoAdvanced SYBR Green Supermix (Bio-Rad). Amplification conditions after an initial denaturation step for 90 s at 95°C were 40 cycles of 95°C, 10 s, for denaturation, 55°C, 10 s, for annealing and 72°C, 30 s, for elongation. GAPDH was used as the reference gene for calculations. Data were analyzed by the 2ΔΔCT threshold cycle method.[21] The forward and reverse primers were used as follows: *GAPDH*, forward: 5′-AATGGATTTGGACGCATTGGT-3′ and reverse: 5′-TTTGCACTGGTACGTGTTGAT-3′; *SNAIL*, forward: 5`-CAGACCCACTCAGATGTCAA-3` and reverse: 5`-CATAGTTAGTCACACCTCGT-3`; Data are expressed as percentages compared with the control.

Statistical treatment of findings was realized by ―Statistics–10v‖ package. Standardize of different indexes was realized by using of: Xn – XΣ / σ, where Xn – individual meaning; XΣ – average value; σ – standard deviation.

RESULTS

Recently increasing interests have been put into tumor-stromal interaction and approaches targeting the tumor stroma of cancer malignancy. EMT plasticity is the pivotal mechanism of cancer stemness in malignancy tumor-stromal interaction. Our study announced the targeted contribution of glioma-related inflammation in EMT plasticity. We have determined the role and potential application of cancer-related inflammation in the EMT induction by exploring the impact of Ca-blocking and anti-inflammatory verapamildrug on the Snail – inducer EMT gene expression level in glioma malignancy patients (n=28). We have performed the peripheral blood cell culture treatment from glioma malignancy patients with different verapamil concentration dilution (1:10; 1:100; 1:1000; 1:10000; from origin verapamil concentration 0.25%) along 72 h under conventional conditions (in Materials and Methods).

We have observed an overexpression of Snail-inducer-EMT gene in glioma malignancy patients which is down-regulated by verapamil drug under culture blood cell treatment of glioma malignancy patients (Figure 1, Table 1).

Interestingly, we have investigated that verapamil induces blood cell polarization by increasing of

transmembrane potential (TMP) which is dramatic reduced in glioma malignancy patients (Figure 2) that correlates with EMT plasticity in cancer progression. We further have studied that verapamiltargeting decreased lymphocytic blast transformation proliferation (LBTP) potential under PHA blood culture treatment of glioma malignancy patients (Figure 3, Figure 4). We have elucidated that LBTP also can correlate with EMT plasticity as we shown during detection of mRNA Snail –inducer EMT gene expression levels with corresponding verapamil concentration dilution (Figure 1). Importantly, we have revealed the oppositive relationship between lymphocytic blast proliferation levels and correspondingly blood cells polarization potential (Figure 5).

Taken together we have revealed the causal interacting between investigated verapamil-dependent anti-inflammatory testing activities as TMP and LBTP and targeting Snail-inducer-EMT under verapamil blood culture treatment in glioma malignancy patients.

Our results therefore demonstrate that verapamil Ca-blocation/anti-inflammatory drug induces a loop of glioma cancer cell stroma - glioma cancer EMT stemness interaction in the tumor microenvironment that promotes glioma malignancy treatment via targeting EMT plasticity during anti-inflammatory therapies.

DISCUSSION

There are emerging current understanding of cancer stem cells (CSCs) in cancer malignancy and metastasis[22] and the clinical application of targeting cancer stemness for cancer treatment.[23] Recently, MSCs (mesenchymal stem cells) as the source of CSCs become a major component of tumor microenvironment.[24,25] Inflammation may play a crucial role in tumor microenvironment formed an inflammatory microenvironment in cancer stemness progression.[16,26,27] Zhao Sun et al. were suggested the role of MSCs in tumor inflammatory microenvironment.[28] Authors have considered the homing of MSCs to tumors from bone marrow or adjacent adipose tissues by the inflammatory mechanism in which tumor-produced inflammatory mediators could attract MSCs from bone marrow or adjacent adipose tissues. Such naïve MSCs as they thought are educated ‘by the tumor inflammatory microenvironment after homing to tumor tissues and transformation to T-MSCs (tumor-mesenchymal stem cells) exert different effects on tumor development.[28] We are considered the alternative mechanism of cancer-related inflammation which has directed MSCs induction in tumor microenvironment throughout epithelial-to-mesenchymal transition (EMT). A growing body of evidence suggests that the EMT plays a management role during tumor malignancy and metastasis and imparts a stemness phenotype and therapeutic resistance to tumor cells.[29] EMT is determined by reprogramming of epithelial cell polarity morphogenesis into mesenchymal invasive cancer stem-like cells with the loss polarity morphogenesis.[30] Therefore, EMT-stemness is becoming the important pathway to cancer stem cell development in cancer malignancy. Regulation of EMT-stemness in tumor inflammatory microenvironment might be the pivotal target for efficacy in the treatment of cancer malignancy. In our study, we determine the role and potential application of cancer-related inflammation in the EMT induction by exploring the impact of anti-inflammatory and Ca-blocking verapamil drug on the Snail – inducer EMT expression level in glioma malignancy patients. The transcription factor Snail is an important EMT induser (Figure 6.[31]).

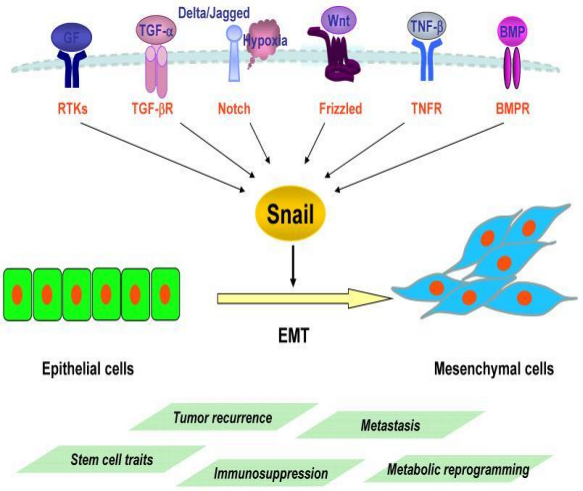


Figure 6. Schematic diagram of the signaling pathways associated with Snail-induced EMT.[31]

Snail directly induces EMT as a key transcriptional repressor of E-cadherin expression.[32] Emerging evidence indicates that Snail confers tumor cells with cancer stem cell-like traits, and promotes drug resistance, tumor recurrence and metastasis.[33,34] Therefore, overexpression of Snail is a biomarker of poor clinical outcome for patients with cancer. We observed that mRNA Snail expression was significantly inhibited in the verapamil treated groups comparing with control groups without verapamil treatment (Figure 1, Table 1). Interestingly, we found that folic acid was tolerant in EMT- Snail gene expression regulation in contrast to down-regulation of Snail by verapamil. We respectively treated the primary periphery blood cells culture from glioma malignancy patients with different verapamil (initial 0.25%) dilution concentration: V10(1:10); V100(1:100); V1000(1:1000); V10000(1:10000) under 72h culturing with phytohemagglutinin (PHA) stimulation in lymphocytic blast transformation proliferation (LBTP) investigation. We have detected significant decreasing LBTP in respectively verapamil culture treatment in glioma IV stage patients (Figure 3, 4). Moreover, we have detected significantly increasing blood cell transmembrane potential (TMP) in respectively verapamil culture treatment in glioma malignancy patients (Figure 2). Transmembrane potential value is of great importance in blood cells aggregation mechanisms. Importantly, we have revealed

the oppositive relationship between lymphocytic blast proliferation levels and correspondingly blood cells polarization potential under verapamil culture blood cell treatment from the glioma malignancy patients (Figure 5). Therefore, we tentatively conclude that verapamil down-regulates EMT- stemness via Snail-signaling pathway inhibition, simultaneously decreasing lymphocytic blast transformation prolipheration (LBTP) and re-activating blood cells polarization as TMP activity in glioma malignancy patients. In addition, earlier, Gridina et al., have been suggested the apparent correlation between plasticity of the transmembrane potential activity (TMP) and DNA stability effect under verapamil treatment in glioma malignancy patients.[35]

Together, on the data obtained we have proposed that glioma malignancy-related inflammation apparently determines EMT-stemness because verapamil as we shown directly induced both anti-inflammatory and anti-EMT actions resulting in decreasing of Snail-inducer-EMT gene expression along with the regeneration of blood cell polarity potential and inhibition of the lymphocytic blast transformation proliferation in glioma malignancy patients.

We have proposed that cancer-related inflammation do not has direct links to cancer genetic instability as have been refered in (Colotta F, et al., 2009), and appearly do not homing MSCs from bone marrow as refered Zhao Sun, et al., 2014, but we have point that cancer-related inflammation has causal link to cancer cell plasticity as EMT-stemness induction in tumor inflammatory microenvironment. Therefore, we are conclused that cancer-associated inflammation is closely related with EMT-stemness mechanism which plays a crucial role in the cancer cell invasion-metastasis phenotype. Accordingly, the inflammatory therapy is becoming more popular in targeted EMT – stemness progression for cancer malignancy treatment and cure.

CONCLUSION

- We are proposed that glioma malignancy-related inflammation activates EMT plasticity.
- We are suggested EMT plasticity via Snail-inducer EMT activation in glioma malignancy patients.
- We are found that verapamil, Ca-blocation and anti-inflammatory drug, repressed EMT plasticity via targeting Snail gene expression in glioma malignancy progression.
- Commonly, our results are promoted ani-inflammatory approaches in targeted EMT stemness prevention in cancer malignancy treatment.

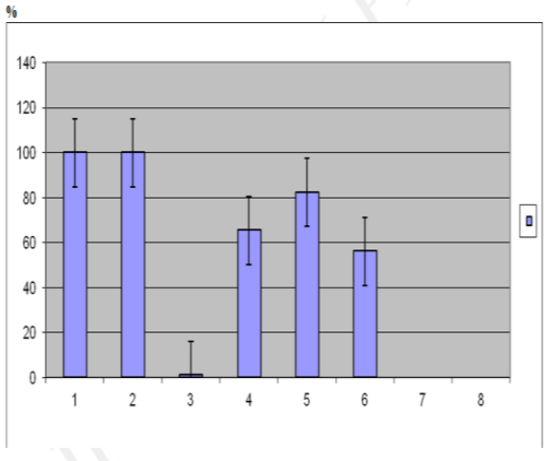


Figure. 1: Decreasing of the level of mRNA Snail gene - EMT-inducer by anti-inflammatory drug verapamil (0.25% verapamil solution) in glioma malignancy patients. (1--control, without verapamil; 2--folic acid, tolerant agent; 3 –verap. dilution 1:10; 4 – verap. dilution1:100; 5--verap. dilution 1:1000; 6 – verap. dilution 1:10000).

Table. 1: Percentage decreasing of mRNA Snail gene expression level by verapamil primary blood culture treatment (72 h).

Anti-inflammatory agentmRNA Snail gene expression (0.25% verapamil, dilution) (percentage %, mean ± SE)	
1.	Control (without verapamil) 100
2.	Folic acid (tolerant agent) 100
3.	Verapamil dilution 1:101,1 ± 0,10
4.	Verapamil dilution 1:10065,38 ± 8,50
5.	Verapamil dilution 1:100082,36 ± 10,30
6.	Verapamil dilution 1:1000056,09 ± 6,17

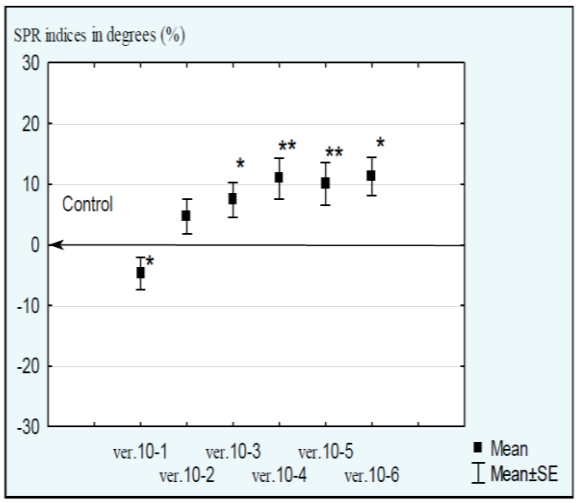
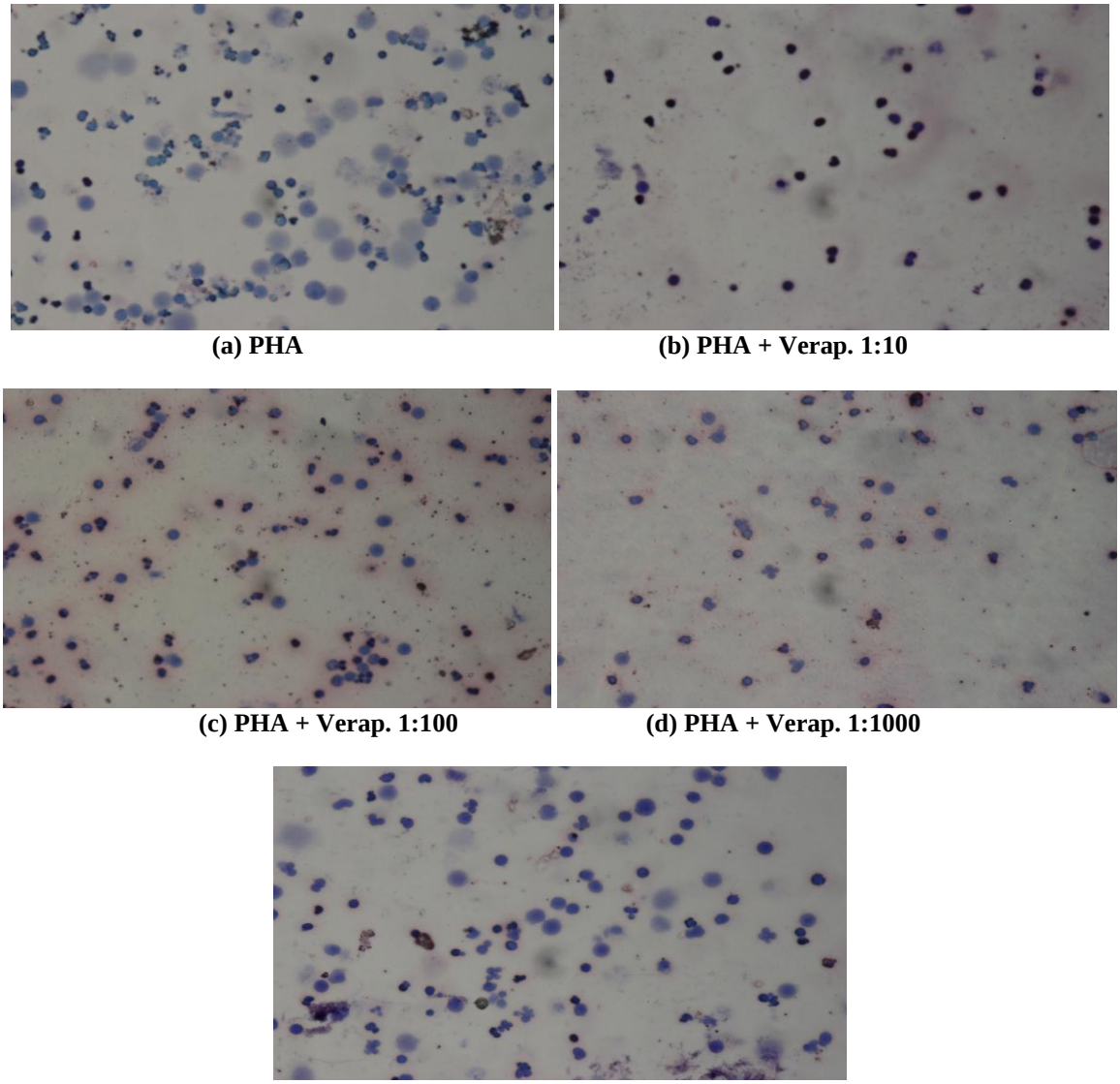


Figure. 2: Effect of different dilution of verapamil anti-inflammatory drug (from 10-1 to 10-6) on the transmembrane potential (TMP) level by SPR blood cells aggregation method in glioma malignancy patients.



(a) PHA (b) PHA + Verap. 1:10

(c) PHA + Verap. 1:100 (d) PHA + Verap. 1:1000

(e) PHA + Verap. 1:10000

Figure. 3: PHA-stimulated lymphocyte blast transformation proliferation (LBTP) of peripheral blood lymphocytes from the IV stage glioma progression patient without and with verapamil culture treatment under 72 h. a) PHA; b) PHA + verap. dilution 1:10; c) PHA + verap. dilution 1:100; d)PHA + verap. dilution 1:1000; e)PHA + verap. dilution 1:10000;

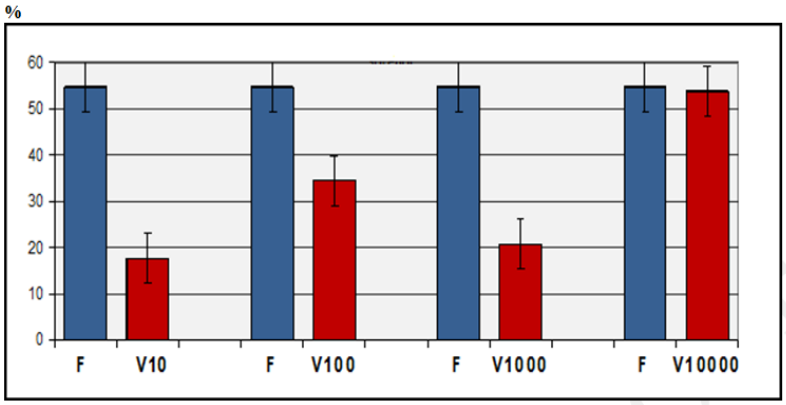


Figure 4. The verapamil concentration-dependent decreasing of lymphocyte blast transformation prolipheration (LBTP) in the primary blood cell culture from the IV stage glioma patients stimulated by PHA under 72 h. (F-control, PHA without verapamil;V10 - PHA+ verap. dilution 1:10; V100 - PHA+verap. dilution 1:100;V1000 - PHA+ verap. dilution 1:1000;V10000 - PHA+verap. dilution 1:10000).

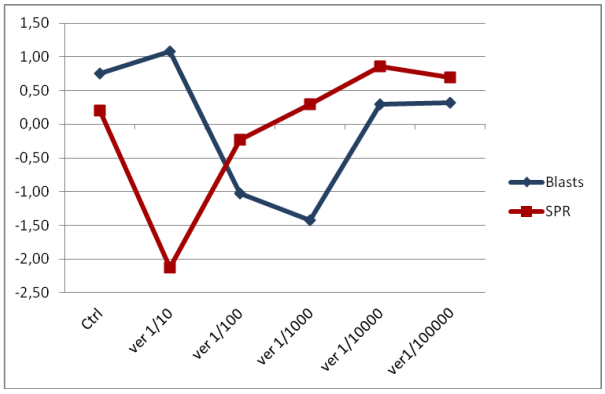


Figure. 5: Anti-inflammatory effects of verapamil in both glioma malignancy inflammatory testings: lymphocytic blast transformation proliferation (LBTP) and blood cells polarity transmembrane potential (TMP) by SPR method assays.

ACKNOWLEDGMENTS

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ABBREVIATIONS

EMT epithelial-mesenchymal transition.
LBTP lymphocytes blast transformation proliferation.
PHA phytohemagglutinin.
TMP transmembrane potential.

SPR surface plasmon resonance phenomenon.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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